BAL1/ARTD9 represses the anti-proliferative and pro-apoptotic IFN -STAT1-IRF1-p53 axis in diffuse large B-cell lymphoma

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Abstract: The B-aggressive lymphoma-1 protein and ADP-ribosyltransferase BAL1/ARTD9 has been recently identified as a risk-related gene product in aggressive diffuse large B-cell lymphoma (DLBCL). BAL1 is constitutively expressed in a subset of high-risk DLBCLs with an active host inflammatory response and has been suggested to be associated with interferon-related gene expression. Here we identify BAL1 as a novel oncogenic survival factor in DLBCL and show that constitutive overexpression of BAL1 in DLBCL tightly associates with intrinsic interferon-gamma (IFN) signaling and constitutive activity of signal transducer and activator of transcription (STAT)-1. Remarkably, BAL1 stimulates the phosphorylation of both STAT1 isoforms, STAT1 and STAT1 , on Y701 and thereby promotes the nuclear accumulation of the antagonistically acting and transcriptionally repressive isoform STAT1 . Moreover, BAL1 physically interacts with both STAT1 and STAT1 through its macrodomains in an ADP-ribosylation-dependent manner. BAL1 directly inhibits, together with STAT1 , the expression of tumor suppressor and interferon response factor (IRF)-1. Conversely, BAL1 enhances the expression of the proto-oncogenes IRF2 and B-cell CLL/lymphoma (BCL)-6 in DLBCL. Our results show for the first time that BAL1 represses the anti-proliferative and pro-apoptotic IFN -STAT1-IRF1-p53 axis and mediates proliferation, survival and chemo-resistance in DLBCL. As a consequence constitutive IFN -STAT1 signaling does not lead to apoptosis but rather to chemo-resistance in DLBCL overexpressing BAL1. Our results suggest that BAL1 may induce an switch in STAT1 from a tumor suppressor to an oncogene in high-risk DLBCL.

DOI: https://doi.org/10.1242/jcs.118174

BAL1/ARTD9 represses the anti-proliferative and pro-apoptotic IFNγ–STAT1–IRF1–p53 axis in diffuse large B-cell lymphoma

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Accepted 2 February 2013
Journal of Cell Science 126, 1969–1980

doi: 10.1242/jcs.118174

Summary

The B-aggressive lymphoma-1 protein and ADP-ribosyltransferase BAL1/ARTD9 has been recently identified as a risk-related gene product in aggressive diffuse large B-cell lymphoma (DLBCL). BAL1 is constitutively expressed in a subset of high-risk DLBCLs with an active host inflammatory response and has been suggested to be associated with interferon-related gene expression. Here we identify BAL1 as a novel oncogenic survival factor in DLBCL and show that constitutive overexpression of BAL1 in DLBCL tightly associates with intrinsic interferon-gamma (IFNγ) signaling and constitutive activity of signal transducer and activator of transcription (STAT)-1. Remarkably, BAL1 stimulates the phosphorylation of both STAT1 isoforms, STAT1α and STAT1β, on Y701 and thereby promotes the nuclear accumulation of the antagonistically acting and transcriptionally repressive isoform STAT1β. Moreover, BAL1 physically interacts with both STAT1α and STAT1β through its macrodomains in an ADP-ribosylation-dependent manner. BAL1 directly inhibits, together with STAT1β, the expression of tumor suppressor and interferon response factor (IRF)-1. Conversely, BAL1 enhances the expression of the proto-oncogenes IRF2 and B-cell CLL/lymphoma (BCL)-6 in DLBCL. Our results show for the first time that BAL1 represses the anti-proliferative and pro-apoptotic IFNγ–STAT1–IRF1–p53 axis and mediates proliferation, survival and chemo-resistance in DLBCL. As a consequence constitutive IFNγ–STAT1 signaling does not lead to apoptosis but rather to chemo-resistance in DLBCL overexpressing BAL1. Our results suggest that BAL1 may induce an switch in STAT1 from a tumor suppressor to an oncogene in high-risk DLBCL.

Key words: BAL1/ARTD9, Diffuse large B-cell lymphoma, Macrodomains, IFNγ-STAT1-signaling, Survival, ADP-ribosylation, IRF1, p53

Introduction

The B-aggressive lymphoma-1 protein and ADP-ribosyltransferase BAL1/ARTD9, here referred to as BAL1, is a nucleocytoplasmic shuttling protein that has been identified as a potential risk-related gene product in diffuse large B-cell lymphoma (DLBCL) (Aguiar et al., 2000; Juszczynski et al., 2006). BAL1 belongs to the diphtheria-toxin-related ADP-ribosyltransferase (ARTD) family (former PARP) of intracellular mono- and poly-ADP-ribosyltransferases (Aguiar et al., 2005; Aguiar et al., 2000; Hottiger et al., 2010). No auto-modification or mono-ADP-ribosyltransferase activity has been observed for BAL1 so far (Aguiar et al., 2005). BAL1 contains two evolutionarily conserved macrodomains. Macrodomains have been recently shown to act as a binding module for free and protein-linked mono- or poly-ADP-ribose (Moyle and Muir, 2010; Timinszky et al., 2009). BAL1 is constitutively expressed in a subset of aggressive chemo-resistant high-risk subtypes of DLBCL, which are associated with an active but ineffective IFNγ-mediated host inflammatory response (HR) (Aguiar et al., 2000; Juszczynski et al., 2006). BAL1 has been suggested to be involved in lymphocyte migration and modulation of IFN-signaling-related gene expression in DLBCL (Juszczynski et al., 2006). However, the exact molecular functions of endogenous BAL1 and its regulatory mechanisms in aggressive DLBCL have not been investigated and remain to be elucidated.

DLBCL is a clinically heterogeneous lymphoid malignancy and the most common subtype of non-Hodgkin's lymphoma in adults, with one of the highest mortality rates (Shaffer et al., 2012). DLBCL has been subdivided into distinct classes (Rosenwald et al., 2002; Shipp et al., 2002). Recently, a high-risk subclass with worse clinical outcomes that is associated with an active but ineffective host inflammatory response has been identified (Monti et al., 2005), known as HR-DLBCL. HR-DLBCL is associated with increased expression of inflammatory mediators and downstream targets of interferon gamma (IFNγ) signaling (Monti et al., 2005). HR-DLBCL lacks most of the common cytogenetic abnormalities and the exact mechanisms of transformation in these tumors remain to be elucidated (Abramson and Shipp, 2005; Monti et al., 2005). The clinical outcome of the HR-DLBCL cluster is not improved, despite the increased inflammatory response (Abramson and Shipp, 2005; Monti et al., 2005). Thus, it has been suggested that either the host immune responses are inhibited by counter-regulatory mechanisms or HR-DLBCL tumors are resistant to chemotherapy, or a combination of both (Abramson and Shipp, 2005; Monti et al., 2005).

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IFNγ exhibits both pro- and anti-tumor properties, depending on the context and cancer type (Dunn et al., 2006). Initially, IFNγ helps protect the host from tumor formation and development (immunosurveillance), but subsequently IFNγ can also promote the tumors to resist the attack (immunoediting) (Dunn et al., 2006; Juszczyński et al., 2008; Lukacher, 2002). The anti-tumor activity of IFNγ is mediated through the signal transducer and activator of transcription 1 (STAT1) and interferon response factor 1 (IRF1) (Taniguchi et al., 2001). Many tumors lack IRF1 or have reduced IRF1 expression levels (Green et al., 1999). Both STAT1 and the gene product of its major target gene *IRF1* have been shown to positively modulate p53-activated apoptotic pathways (Taniguchi et al., 2001; Townsend et al., 2004). Remarkably, recent studies provided evidence that STAT1 can also act as a proto-oncogene product in solid cancers (Khodarev et al., 2004). However, the exact molecular mechanisms of how STAT1 acts as an oncogene are not yet known.

We have identified BAL1 as a novel co-repressor for the transcriptional repression of tumor suppressor IRF1 and a co-activator for the transcriptional activation of the proto-oncogenes *IRF2* and B-cell CLL/lymphoma 6 (*BCL6*). BAL1 interacts with the IFNγ receptor (IFNGR) complex and enhances tyrosine phosphorylation of both isoforms of STAT1 on Y701 and their subsequent nuclear translocation, thereby promoting the nuclear accumulation of the antagonistically acting and transcriptionally repressive isoform STAT1β. BAL1 interacts with both STAT1 isoforms through its macrodomains in an ADP-ribosylation-dependent manner, and together with STAT1β inhibits the *IRF1* promoter. BAL1 counteracts the IFNγ-dependent anti-proliferative and pro-apoptotic IFNγ–STAT1–IRF1–p53 axis and as a consequence, mediates proliferation, chemo-resistance and survival in high-risk DLBCL.

Results

BAL1 is constitutively expressed in DLBCL and is associated with aberrant IFNγ/STAT1 signaling

Previous reports showed that BAL1 is constitutively overexpressed in high-risk primary DLBCL with an active host inflammatory response (Aguiar et al., 2005; Aguiar et al., 2000; Juszczynski et al., 2006). Overexpression of BAL1 has also been observed in the aggressive ABC-DLBCL cell lines OCI-Ly3 and OCI-Ly10 as well as in the GCB-HR-like DLBCL cell line SUDHL7 (Aguiar et al., 2005; Aguiar et al., 2000; Juszczynski et al., 2006). IFNγ/STAT1–IRF1 signaling has been shown to stimulate the expression of BAL1 *in vitro* (Juszczynski et al., 2006; Shi et al., 2011). IFNγ signaling is mediated through activation of the IFNγ receptor and Janus kinases (JAK) 1 and 2 that lead to tyrosine phosphorylation of STAT1 on Y701, homodimerization and translocation of STAT1 to the nucleus where it induces the transcription of IFNγ-stimulated genes such as *IRF1* (Dunn et al., 2006). In order to confirm these data and to investigate whether constitutive expression of endogenous BAL1 is associated with constitutively active STAT1 signaling and IRF1 expression we analyzed STAT1 activity in different BAL1-expressing and -non-expressing DLBCL cell lines including the chemo-resistant GCB-related HR-DLBCL cell line SUDHL7.

Indeed, our immunoblot analysis of BAL1, STAT1, pSTAT1, STAT2, pSTAT2 and IRF1 expression, revealed that constitutively expressed BAL1 is tightly associated with intrinsic IFNγ signaling and constitutively active STAT1 (Fig. 1A,B). Moreover, our STAT1-knockdown analysis using siRNA revealed that constitutive expression of BAL1 is strictly dependent on the transcriptional activity of STAT1 (Fig. 1C). Subsequent chromatin immunoprecipitation analysis revealed that endogenous STAT1 is recruited to the *BAL1* promoter (Fig. 1D), strongly indicating that STAT1 directly activates *BAL1* gene expression. STAT2 activity appears to be normal in all DLBCL cell lines tested and not involved in upregulation of BAL1 in HR-DLBCL cells, demonstrated by the absence of phosphorylated STAT2. The observed IFNγ-induced BAL1 expression tightly correlates with the induced activity of STAT1 in the ABC-DLBCL cell lines OCI-Ly3 and OCI-Ly10. However, the lower expression levels of BAL1 in OCI-Ly3 and OCI-Ly10 could also be mediated through the constitutively high activity of NF-κB family members in these ABC DLBCL cell lines (Davis et al., 2010; Ngo et al., 2011; Shaffer Iii et al., 2012). Surprisingly, tumor suppressor IRF1, a major target of STAT1 (Taniguchi et al., 2001), is strongly downregulated in the presence of both constitutively and inducibly expressed BAL1, whereas it is upregulated in the absence of constitutively expressed BAL1 (Fig. 1B). These observations indicate that BAL1 might act as a transcriptional repressor of the *IRF1* gene.

Interestingly, our analysis of primary DLBCL tumors revealed that 18% of the primary tumor samples analyzed showed positive nuclear staining for all three analyzed markers IRF2, STAT1-pY701 and STAT1-pS727, whereas benign tissue did not show this pattern (Fig. 1E; supplementary material Fig. S1D,E).

BAL1 inhibits tumor suppressor IRF1 and activates the proto-oncogene BCL6 to mediate proliferation

In order to test whether BAL1 inhibits the tumor-suppressing IFNγ–STAT1–IRF1 axis and thus directly stimulates cell proliferation, we first analyzed the proliferation of SUDHL7 cells in which BAL1 had been knocked down (SUDHL7-shBAL1) and control cells (SUDHL7-shmock). Remarkably, this experiment revealed that knockdown of BAL1 strongly inhibits proliferation (Fig. 2A; supplementary material Fig. S2A). We next investigated whether BAL1 is directly required for the transcriptional downregulation of tumor suppressor genes and upregulation of proto-oncogenes involved in proliferation and survival in DLBCL. We first analyzed the expression levels of the IFNγ/STAT1-dependent tumor suppressors and oncogene products such as STAT1, IRF1 and IRF2. Our results demonstrate that the expression of tumor suppressor IRF1 is strongly upregulated in BAL1-knockdown cells, whereas the expression level of the proto-oncogene IRF2 is strongly reduced (Fig. 2B,C). In contrast, the expression of STAT1 and its isoforms is not regulated by BAL1 in SUDHL7 (Fig. 2C; supplementary material Fig. S2B). STAT1 exists in two mayor isoforms, the full-length isoform STAT1α, which mainly acts as a sequence-specific activator of gene expression and STAT1β, lacking a complete transactivation domain and acting as a transcriptional repressor and antagonist of STAT1α (Baran-Marszak et al., 2004; Zakharova et al., 2003). Remarkably, our expression analysis demonstrates that BAL1 also stimulates the expression of another IFNγ/STAT1-independent crucial proto-oncogene *BCL6*, while concomitantly repressing the tumor-suppressor PR-domain-containing 1 (*Prdm1*) gene, the gene product of which is the B-lymphocyte-induced-maturation protein-1 (BLIMP1), an antagonist of BCL6 (Fig. 2B,C). The observed transcriptional upregulation of *BCL6* by BAL1 is also consistent with previous reports demonstrating that subsets of high-risk DLBCL are dependent on BCL6 (Saito et al., 2009).

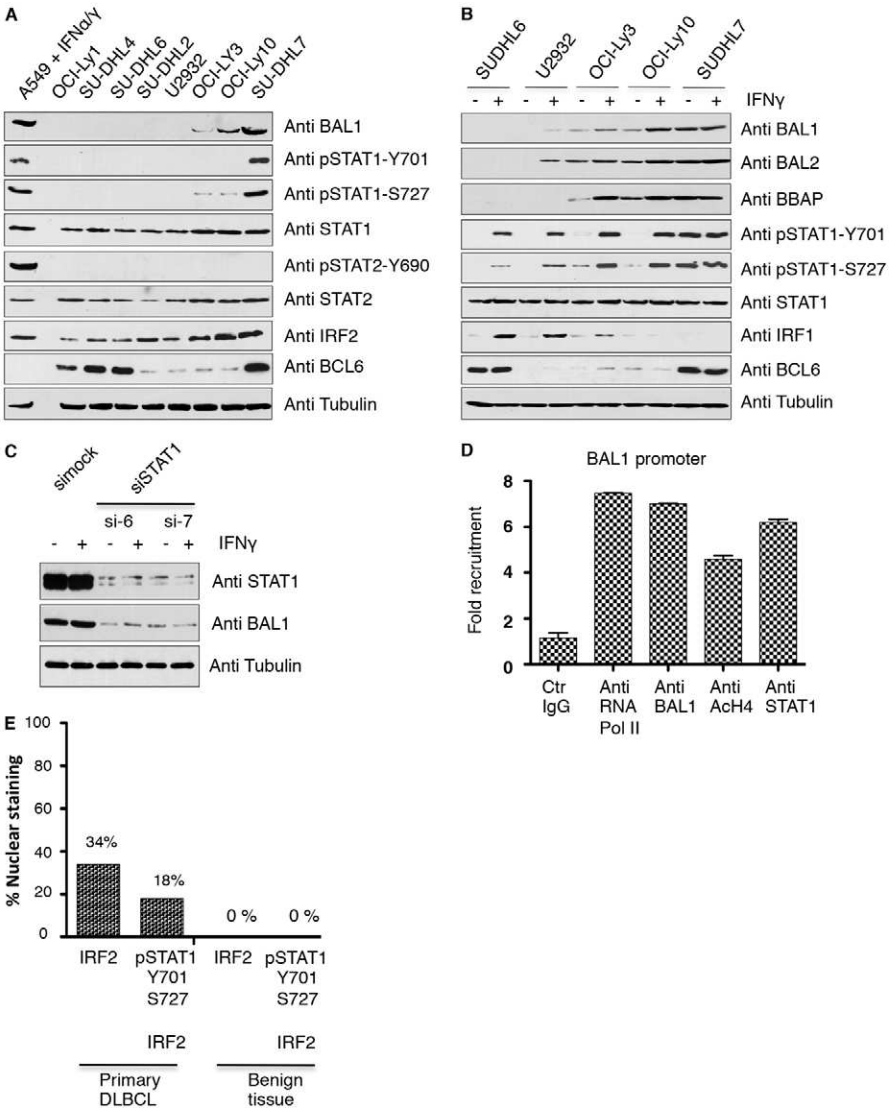

Fig. 1. BAL1 is constitutively expressed in DLBCL associated with constitutively active STAT1 signaling. (**A**) Immunoblot analysis of untreated GCB-DLBCL cell lines (OCI-Ly1, SUDHL4 and SUDHL6), ABC-DLBCL cell lines (SUDHL2, U2932, OCI-Ly3 and OCI-Ly10) and GCB-HR-DLBCL cell line SUDHL7. Whole-cell extracts were separated by SDS-PAGE, blotted and subsequently probed with antibodies for STAT1, pSTAT1(Y701), pSTAT1(S727), STAT2, pSTAT2(Y690), BAL1, IRF2, BCL6 and tubulin. (**B**) Immunoblot analysis of STAT1 signaling in GCB-, ABC- and HR-GCB-DLBCL cell lines untreated or treated with 1000 U/ml IFNγ for 8 hours. GCB-DLBCL cell line (SUDHL6), ABC-DLBCL cell lines (U2932, OCI-Ly3 and OCI-Ly10) and the GCB-HR-DLBCL cell line SUDHL7 were untreated or treated with IFNγ for 8 hours and then whole-cell extracts separated by SDS-PAGE and subsequently probed with antibodies for BAL1, BAL2/ARTD8, BBAP/DTX3L STAT1, pSTAT1(Y701), pSTAT1(S727), IRF1, BCL6 and tubulin. (**C**) Immunoblot analysis of BAL1 and STAT1 expression using whole-cell extracts of SUDHL7-simock and siSTAT1-knockdown cells untreated or treated with 1000 U/ml IFNγ for 8 hours. (**D**) Chromatin immunoprecipitation (ChIP) analysis of the *BAL1* promoter for H4K16-acetylation, STAT1, BAL1 and RNA-Pol-II recruitment in SUDHL7 cells, using anti-STAT1, anti-BAL1, anti RNA-Pol-II, anti-H4K16Ac and control (Ctr) antibodies. (**E**) Analysis of primary DLBCL tumors: the bars indicate the percentage of positive nuclear staining for IRF2 alone and for all three analyzed markers IRF2, pSTAT1(Y701) and pSTAT1(S727) in primary DLBCL tumors and benign samples. For a detailed description of the scoring system see Materials and Methods and supplementary material Tables S3, S4.

BCL6 is overexpressed in the majority of patients with aggressive DLBCL (Saito et al., 2009). Constitutive expression of BCL6 mediates lymphomagenesis through aberrant proliferation and cell survival (Saito et al., 2009). Remarkably, BAL1 also enhances the expression of BAL2/ARTD8 and the BAL2/ARTD8 target, the oncogenic PIM1 kinase (Cho et al., 2009a) (Fig. 2B). BAL2/ARTD8 is another macrodomain-containing ARTD family member (Cho et al., 2009b; Goenka and Boothby, 2006; Goenka et al., 2007).

Next we tested whether BAL1 directly regulates *BCL6* and *IRF1* gene expression. Indeed chromatin immunoprecipitation analysis of SUDHL7 cells revealed that endogenous BAL1 is recruited to both the STAT1-dependent *IRF1* promoter and to the STAT1-independent *BCL6* promoter (Fig. 2D), strongly indicating that BAL1 directly repress *IRF1* gene expression and stimulates *BCL6* gene expression on the level of transcriptional activation (Fig. 2D). Activation of BCL6 tightly correlates with H4K16-acetylation and RNA-PolII recruitment, whereas repression of IRF1 correlates with absence of H4K16 acetylation and RNA-PolII recruitment (Fig. 2D). In contrast, activation of PIM1 is not directly mediated by *BCL1* (supplementary material Fig. S2D). As expected, STAT1 is not required for the recruitment of BAL1 to the *BCL6* promoter.

BAL1 inhibits the IRF1-mediated cell death and activates BCL6-mediated survival

In order to test whether the observed stimulation of BCL6 expression and repression of IRF1 by BAL1 also has direct effects on cell survival we treated SUDHL7 BAL1-knockdown and sh-mock control cells with etoposide and/or doxorubicin and subsequently analyzed the survival and proliferation as well as gene expression (Fig. 3A–C). Remarkably, these experiments revealed that BAL1 not only blocks IFNγ–STAT1–IRF1-mediated apoptosis and inhibition of growth, but also reverses the chemo-resistance of SUDHL7 (Fig. 3A,B). Absence of BAL1 strongly increases the expression of pro-apoptotic and anti-proliferative gene products including p21, BAD, p53 or CASP3 while simultaneously downregulating the expression of pro-survival gene products such BCL2 or BCL-XL (Fig. 3B). IRF1 has been shown to induce both ligand-dependent (extrinsic) and ligand-independent (intrinsic) caspase-mediated apoptosis (Stang et al., 2007). Recent studies demonstrated that IRF1 inhibits the expression of pro-survival members of the BCL2 family and induces the expression and activation of pro-apoptotic and anti-proliferative gene products, including p53, p21 and CASP3

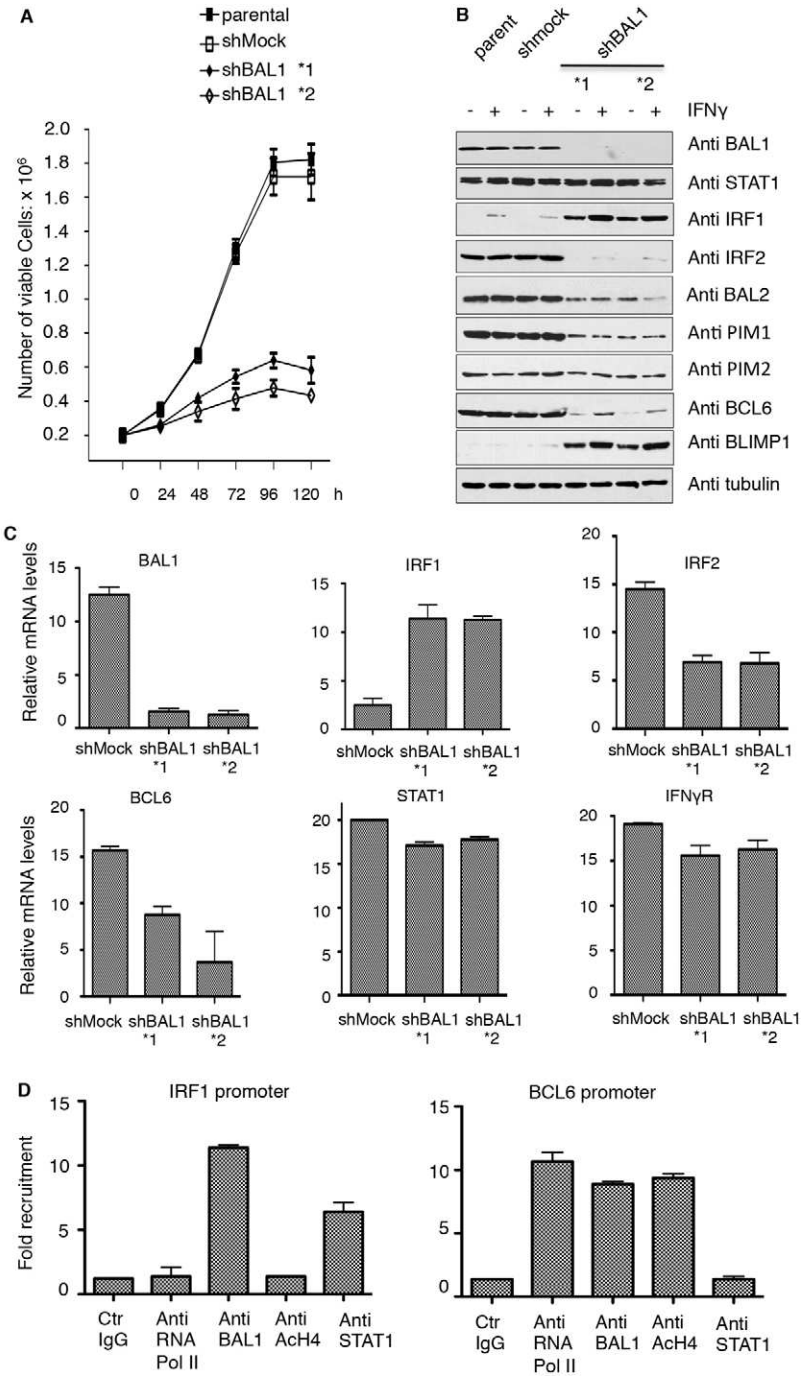

Fig. 2. BAL1 inhibits IRF1 and activates BCL6 to mediate proliferation in DLBCL. (**A**) Cell proliferation analysis of SUDHL7-parental, stable SUDHL7-shmock and BAL1-knockdown cells over 120 hours, was assessed by a Trypan Blue exclusion assay. Cells were seeded at 0.2×10^6 cells/ml in triplicate in six-well dishes and counted every day for 5 days. Values are means ± s.d. from three independent experiments performed in triplicate. (**B**) Immunoblot analysis of tumor suppressor gene and proto-oncogene products. Parental wild-type SUDHL7, shmock-RNA and *BCL1*-shRNA knockdown cells were untreated or treated with 1000 U/ml IFNγ for 8 hours and then whole-cell extracts separated by SDS-PAGE, blotted and subsequently probed with antibodies for BAL1, BAL2, STAT1, IRF1, IRF2, BCL6, BLIMP1, PIM1, PIM2 and tubulin. (**C**) Gene expression analysis of stable SUDHL7 shmock RNA and *BCL1*-shRNA knockdown cells. mRNAs were isolated from transient SUDHL7 shRNA knockdown cells and *BAL1*, *STAT1*, *IRF1*, *IRF2*, *IFNGR2* and *BCL6* mRNA levels were measured by qPCR and normalized against *GAPDH*. (**D**) ChIP analysis of *IRF1* and *BCL6* promoters for H4K16-acetylation, STAT1, BAL1 and RNA-Pol-II recruitment in SUDHL7 cells using anti-STAT1, anti-BAL1, anti RNA-Pol-II, anti-H4K16Ac and control (Ctr) antibodies.

(Choo et al., 2006; Schwartz et al., 2011; Shi et al., 2011). Conversely, it has been previously demonstrated that BCL6 can also suppress both the basal and the induced expression levels of tumor-suppressor genes *PRDM1/BLIMP1* and *p53*, the cyclin-dependent kinase inhibitor gene *p21* as well as the anti-apoptotic proto-oncogene *BCL2* in DLBCL (Phan and Dalla-Favera, 2004; Saito et al., 2009). Interestingly, phosphorylation and inactivation of the pro-apoptotic protein BAD is strongly reduced in absence of BAL1 (Fig. 3B). However, phosphorylation of BAD at S112 is probably not directly regulated by BAL1 but rather through BAL2 and its target, the oncogenic kinase PIM1. Several studies showed that phosphorylation of BAD on serine 112 is also mediated by PIM1 and contributes to cell survival in B-cell lymphoma (Aho et al., 2004; Chen et al., 2008; Yan et al., 2003).

The observed concomitant overexpression of BCL2 and BCL6 in SUDHL7 cells also indicates that BAL1 blocks BCL6-mediated repression of the *BCL2* gene, which is frequently disrupted in DLBCL (Saito et al., 2009). We next investigated whether the siRNA-mediated knockdown of STAT1 could inhibit the pro-apoptotic and/or anti-proliferative pathways in absence of BAL1 and thus could rescue proliferation and/or survival in these cells. These experiments revealed that siRNA-mediated knockdown of STAT1 in SUDHL7 cells indeed blocks the IRF1-mediated pro-apoptotic and anti-proliferative pathways (Fig. 3D–F). However,

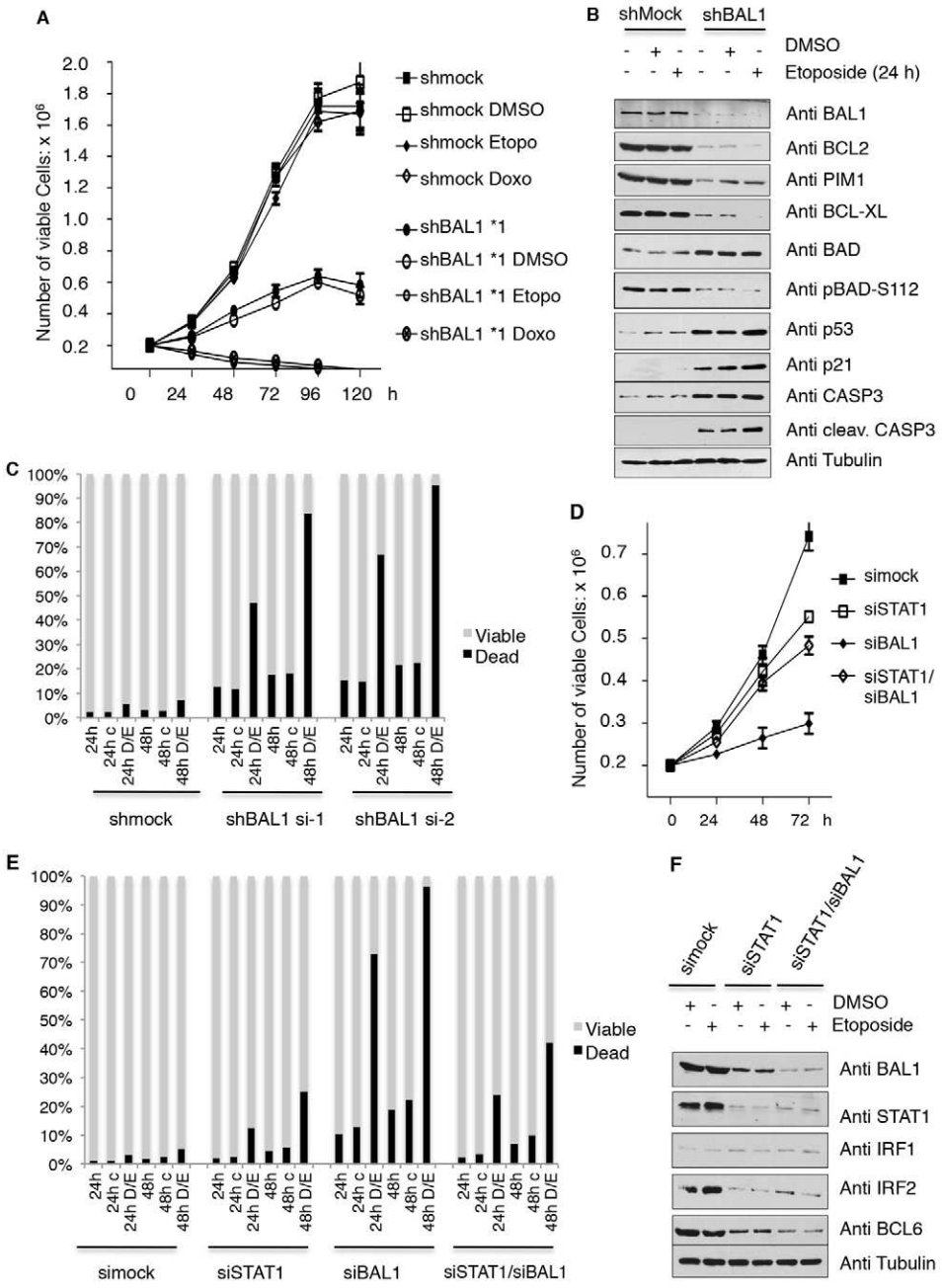

Fig. 3. BAL1 inhibits the IRF1-mediated pro-apoptotic pathways and activates the BCL6-mediated survival pathways. (**A**) Cell proliferation analysis of parental SUDHL7 cells, stable SUDHL7 shmock and BAL1-knockdown cells treated with etoposide (Etopo; 25 μM) and/or doxorubicin (Doxo; 5 μM) was assessed by a Trypan Blue exclusion assay. Cells were seeded at 0.2×10^6 cells/ml in triplicate in six-well dishes, treated as indicated and counted every day for 5 days. Values are means ±s.d. from three independent experiments performed in triplicate. (**B**) Immunoblot analysis of pro-apoptotic and survival factors. SUDHL7 shmock-RNA and BAL1-shRNA knockdown cells were untreated or treated with Etoposide (25 μM) for 24 hours and then whole-cell extracts separated by SDS-PAGE, blotted and subsequently probed with antibodies for BAL1, BCL2, BCL-xL, PIM1, BAD, pBAD-S112, p53, p21, Casp3, cleaved Casp3 and tubulin. (**C**) Cell viability analysis of parental SUDHL7 cells, stable SUDHL7 shmock and BAL1-knockdown cells untreated (control, c) treated with etoposide (E; 25 μM) and/or doxorubicin (D; 5 μM) was assessed by a Trypan Blue exclusion assay. Cells were seeded at 0.2×10^6 cells/ml in triplicate in six-well dishes, treated as indicated and counted every day for 5 days. Values are mean percentages from three independent experiments performed in triplicate. (**D**) Cell proliferation analysis of untreated SUDHL7-si mock, siBAL1, siSTAT1 and siSTAT1/BAL1 knockdown cells. (**E**) Cell viability analysis of SUDHL7-simock, siBAL1, siSTAT1 and siBAL1/siSTAT1-knockdown cells untreated (control, c) or treated with etoposide (25 μM) and doxorubicin (5 μM) was assessed by a Trypan Blue exclusion assay. Cells were seeded at 0.2×10^6 cells/ml in triplicate in six-well dishes, treated as indicated and counted every day for 5 days. Values are means from three independent experiments performed in triplicate. (**F**) Immunoblot analysis of siSTAT1 knockdown cells. SUDHL7 simock-RNA, STAT1-siRNA and STAT1-siRNA/BAL1-siRNA knockdown cells were untreated or treated with Etoposide (25 μM) for 24 hours and then whole cell extracts separated by SDS-PAGE, blotted and subsequently probed with antibodies for STAT1, BAL1, IRF1, IRF2 and BCL6.

the observed proliferation defects and increased cell death in siBAL1 cells could not be fully rescued in presence of siSTAT1 RNA, indicating first that STAT1 itself is required for survival and proliferation, most probably through IRF2, and second, the presence of overexpressed BCL6 is equally important for survival in this cell line (Fig. 3D–F).

BAL1 forms complexes with STAT1α and STAT1β through its macrodomains and is recruited by STAT1 to its target promoters

Since tumor suppressor IRF1 is a major target of STAT1 we investigated whether BAL1 could form a complex with STAT1 or other IFN-related STAT members *in vivo*. We first performed co-immunoprecipitation experiments with HA-tagged BAL1 and FLAG-tagged STAT1-6 transiently coexpressed in HEK293 cells, as well as with endogenously expressed BAL1 and STAT1 in SUDHL7 cells. Our interaction studies revealed that BAL1 specifically interacts with both STAT1 and STAT2 (Fig. 4A), indicating that endogenous BAL1 forms complexes with both IFNγ-induced STAT1 homodimers (Fig. 4B). Moreover, BAL1 interacts with both STAT1α and STAT1β isoforms (Fig. 4C). No interaction between BAL1 and other STATs (STAT3–6) could be observed under the tested conditions (Fig. 4A). We next tested which domain of BAL1 is required for the observed interaction by performing co-immunoprecipitation experiments with HA-tagged full-length BAL1 and deletion mutants coexpressed together with FLAG-tagged STAT1 or STAT2 in HEK293 cells. Remarkably, these mapping

experiments revealed that the observed interactions between BAL1 with STAT1 are mediated through both macrodomains (Fig. 4D; supplementary material Fig. S3A) and thus might be dependent on n-ADP-ribosylation. Subsequent co-immunoprecipitation experiments revealed that the interaction is indeed mediated by (mono)-ADP-ribosylation (Fig. 4E). Thus, our results suggest a potential regulatory connection between ADP-ribose binding modules and mono-ADP-ribosylation-dependent signaling and gene expression in high-risk DLBCL and other B-cell lymphomas. STAT1 is modified *in vitro* by BAL2/ARTD8 and ARTD10 (data not shown). However, we have found no evidence so far that STATs are ADP-ribosylated *in vivo*, and thus we were unable to elucidate the exact mechanisms *in vivo*. Analyzing protein mono-ADP-ribosylation *in vivo* is a difficult task because antibodies specifically recognizing mono-ADP-ribosylated glutamate or arginine residues are lacking and ADP-ribosylation of proteins *in vivo* is not easily analyzed by mass spectrometry (Hottiger et al., 2010). Moreover, there are no ARTD-family-member-specific ARTD/PARP inhibitors available that would specifically target BAL2, BAL3 or other

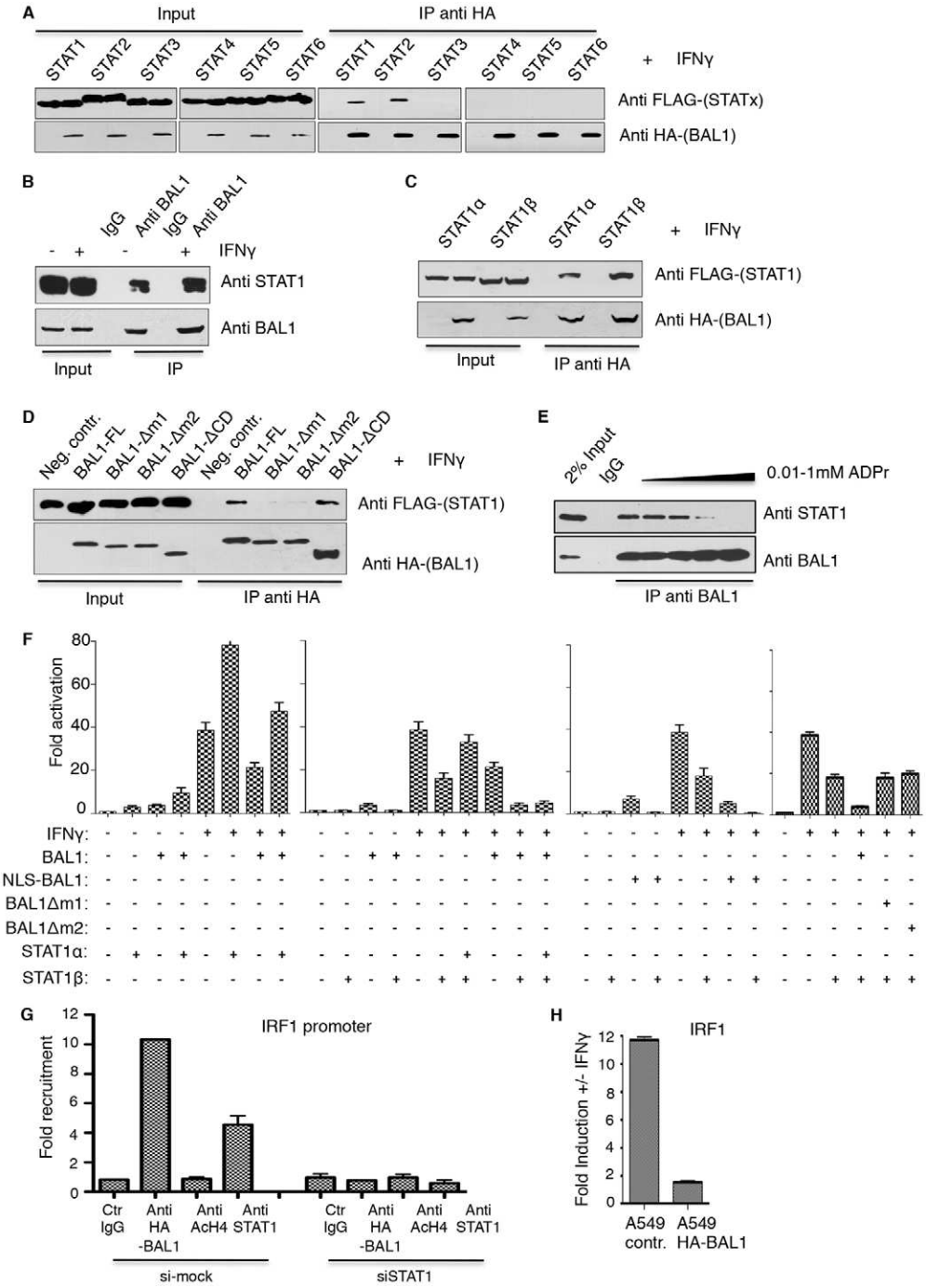

Fig. 4. See next page for legend.

mono-ADP-ribosylating ARTD family members (Hottiger et al., 2010; Wahlberg et al., 2012). We could not observe any effects on STAT1-dependent signaling and gene expression using Olaparib or Veliparib, two ARTD/PARP inhibitors highly specific to ARTD1 and ARTD2 (data not shown), also indicating that the enzymatic activity of ARTD1 and/or ARTD2 is not required for these BAL1/ARTD9-dependent processes.

Recent studies showed that overexpression of STAT1β, the antagonistic isoform of STAT1α increases the growth rate of cells and their resistance to drug-induced apoptosis and cell cycle arrest by repressing STAT1α target genes such as *p21* and *IRF1* in human B cells (Baran-Marszak et al., 2004). We therefore performed *IRF1*-promoter-driven luciferase reporter assays with BAL1, STAT1α and STAT1β, in the BAL1-negative DLBCL cell line U2932 (Fig. 4F). Indeed, coexpression of BAL1 wild-type or macrodomain deletion mutants together with STAT1α or STAT1β, respectively, along with an *IRF1*-promoter-driven luciferase reporter in U2932 cells showed that overexpression of BAL1 wild-type together with STAT1β synergistically downregulated the *IRF1*-promoter-driven luciferase reporter upon IFNγ stimulation, even in presence of STAT1α, whereas no BAL1-mediated repression was observed with BAL1-macrodomain deletion mutants, suggesting that the observed inhibitory effect is directly mediated through the macrodomain-dependent interaction of BAL1 with STAT1β (Fig. 4F). Next we tested whether STAT1 is required for the recruitment of BAL1 to its STAT1-dependent target promoters. Indeed chromatin immunoprecipitation analysis in A549 cells ectopically expressing HA-tagged BAL1 independent of STAT1 (supplementary material Fig. S3B,C) revealed that the recruitment of BAL1 to the *IRF1* promoter is strictly dependent on STAT1 (Fig. 4G,H).

Fig. 4. BAL1 forms an ADP-ribosylation-dependent complex with STAT1 and represses the IRF1 promoter together with STAT1β. (**A**) Co-immunoprecipitation of BAL1 and STATs overexpressed in HEK293 cells. HEK293 cells were co-transfected with expression vectors for HA-tagged BAL1 full length along with FLAG-tagged STAT1, STAT2, STAT3, STAT4, STAT5 or STAT6 and subsequently stimulated for 1 hour with 1000 U/ml IFNγ. HA–BAL1 and STAT1 complexes were then co-immunoprecipitated, separated on SDS-PAGE, blotted and subsequently probed with antibodies for HA (BAL1) and FLAG tag (STATs). (**B**) Interaction of endogenous BAL1 and STAT1 is partially dependent of IFNγ. SUDHL7 cells were stimulated for 1 hour with 1000 U/ml IFNγ and endogenous BAL1 and STAT1 complexes subsequently co-immunoprecipitated using an anti-BAL1 antibody. Complexes were then separated on SDS-PAGE, blotted and probed with antibodies against endogenous BAL1 and STAT1. (**C**) BAL1 interacts with both isoforms of STAT1. HEK293 cells were co-transfected with expression vectors for HA-tagged BAL1 full length along with FLAG-tagged STAT1α or STAT1β and subsequently stimulated for 1 hour with 1000 U/ml IFNγ. HA–BAL1 and STAT1 complexes were then co-immunoprecipitated, separated on SDS-PAGE, blotted and probed with antibodies for HA (BAL1) and FLAG tag (STATs). (**D**) Mapping of the interaction domains in BAL1. HEK293 cells were co-transfected with expression vectors for HA-tagged BAL1 full length, or deletion mutants [deletion of macro domain 1 and 2 (Δm1 and Δm2) or catalytic domain (ΔCD)] along with FLAG-tagged STAT1, and subsequently stimulated for 1 hour with 1000 U/ml IFNγ. HA–BAL1 and STAT1 complexes were then co-immunoprecipitated, separated on SDS-PAGE, blotted and probed with antibodies for HA (BAL1) and FLAG tag (STAT1). (**E**) Co-immunoprecipitation of endogenous BAL1 and STAT1 in the presence of ADP-ribose. SUDHL7 cells were stimulated for 1 hour with 1000 U/ml IFNγ and endogenous BAL1 and STAT1 complexes were subsequently co-immunoprecipitated in the presence of increasing concentrations of ADP-ribose (0.01–1 mM) using an anti-BAL1 antibody. BAL1-STAT1 complexes were then separated on SDS PAGE, blotted and subsequently probed with antibodies against endogenous BAL1 and STAT1. (**F**) BAL1 inhibits the *IRF1*-promoter-driven luciferase in U2932 cells. BAL1-negative U2932 cells were seeded in 12-well dishes at 0.4×10^6 cells/ml and co-transfected with an *IRF1*-promoter-driven luciferase reporter vector (500 ng DNA/ml) along with expression vectors for BAL1 wild type, 3x-NLS-BAL1 wild type or BAL1-macrodomain deletion mutants (BAL1Δm1 or BAL1Δm2), STAT1α (first and middle left panels) or STAT1β (middle left, middle right and right panels; 800 ngDNA/ml) and with the control reporter plasmid, pRL-hTK (100 ng/ml; TK-Renilla–luciferase control, Promega). The cells were then treated with 1000 U/ml IFNγ for 10 hours or left untreated. *IRF1*-promoter-Luciferase activities are normalized to the luciferase activities of the internal TK-Renilla–luciferase control and presented as means from 5 independent experiments performed in triplicate ± standard deviations. (**G**) ChIP analysis of *IRF1* promoter for H4K16-acetylation, STAT1, HA–BAL1 and RNA-Pol-II recruitment in si-mock- and siSTAT1-RNA-treated A549 knockdown cells ectopically expressing HA-tagged BAL1 using anti-STAT1, anti-HA, anti RNA-Pol-II, anti-H4K16Ac and control (Ctr) antibodies. (**H**) *IRF1* gene expression analysis in A549 control cells and A549 cells ectopically expressing HA-tagged BAL1. *IRF1* mRNA levels were measured by qPCR and normalized against *GAPDH*.

BAL1 stimulates the phosphorylation and nuclear translocation of STAT1

We next investigated whether BAL1 could modulate the balance between transcriptionally active STAT1α and transcriptionally repressive STAT1β complexes in the nucleus through stimulation of STAT1α and STAT1β phosphorylation on tyrosine 701. Indeed, our phosphorylation analysis revealed that BAL1 stimulates the phosphorylation of both STAT1α and STAT1β on Y701 but not on serine 727 of the transcriptionally activating isoform STAT1α (Fig. 5A). IFNGR–JAK2-mediated phosphorylation of STAT1 on Y701 is required for STAT1 dimerization and its nuclear translocation (Darnell et al., 1994; Mowen and David, 2000). Phosphorylation on Y701 also enhances the nuclear shuttling by triggering the nuclear retention of the shuttling STAT1α and STAT1β, which are kept in the nucleus until tyrosine dephosphorylation occurs (Meyer et al., 2003). JAK2 is thought to phosphorylate STAT1 on Y701 in the cytoplasm, whereas JAK1 seems to be required for phosphorylation on Y701 in the nucleus, preventing nuclear export of STAT1 (Meyer et al., 2003; Mowen and David, 2000). However, phosphorylation on S727 in the transactivation domain of STAT1α can also occur independently of STAT1 tyrosine phosphorylation (Decker and Kovarik, 2000).

BAL1 might, therefore, mainly influence the shuttling kinetics of STAT1. Our subsequent subcellular fractionation analysis confirmed that BAL1 is indeed required for the constitutive exclusively nuclear localization of STAT1 in HR-DLBCL (Fig. 5B). Finally, our co-immunoprecipitation analysis revealed that BAL1 interacts with the IFNGR1 complex but not with the pY701-specific tyrosine phosphatase PTBN1 in HR-DLBCL (Fig. 5C), indicating that BAL1 may directly stimulate JAK2-mediated phosphorylation of STAT1 and thereby promote the nuclear accumulation of the antagonistically acting and transcriptionally repressive isoform STAT1β. Remarkably, a recent study provided the first evidence that the BAL1-related BAL2/ARTD8 promotes the survival of myeloma cells by inhibiting the kinase activity of c-Jun N-terminal kinase 1 (JNK1) (Barbarulo et al., 2012). Since phosphorylation of STAT1α on S727 is not affected by BAL1, our results indicate that BAL1 influences the nuclear activities of the transcriptionally repressive isoform STAT1β thereby tipping

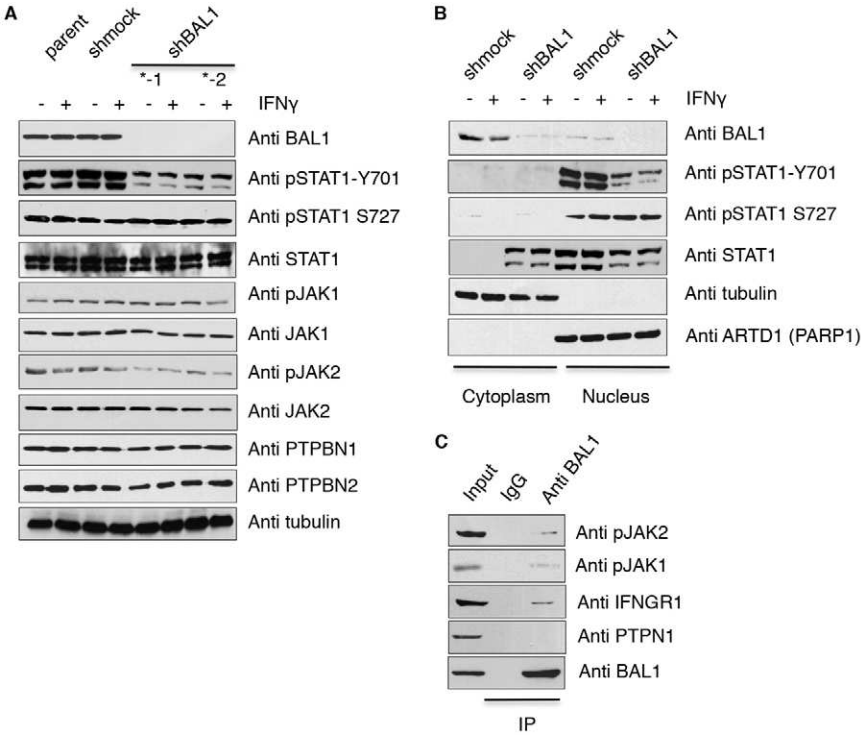

Fig. 5. BAL1 specifically stimulates the phosphorylation of STAT1α and STAT1β on Y701. (**A**) Immunoblot analysis of STAT1 signaling in SUDHL7-shmock and -BAL1-knockdown cells. Parental wild-type SUDHL7, shmock-RNA and *BAL1*-shRNA knockdown cells were untreated or treated with 1000 U/ml IFNγ for 8 hours and then whole-cell extracts were separated by SDS-PAGE, blotted and probed with antibodies for BAL1, STAT1, pSTAT1(Y701), pSTAT1(S727), JAK1, pJAK1, JAK2, pJAK2, PTPN1, PTPN2 and tubulin. (**B**) Subcellular fractionation analysis of STAT1 signaling in SUDHL7-shmock and BAL1-shRNA knockdown cells. The cells were untreated or treated with 1000 U/ml IFNγ for 8 hours and then nuclear and cytoplasmic cell extracts were separated by SDS-PAGE, blotted and probed with antibodies for BAL1, STAT1, pSTAT1(Y701), pSTAT1(S727), ARTD1(PARP1) and tubulin. (**C**) Co-immunoprecipitation analysis of endogenous BAL1-tyrosine-kinase/-phosphatase complexes in SUDHL7 cells. Endogenous BAL1/IFNGR complexes were co-immunoprecipitated using an anti-BAL1 antibody. Complexes were then separated by SDS-PAGE, blotted and probed with antibodies against endogenous BAL1, IFNγR1, pJAK1, pJAK2 and PTPN1.

the antagonistic balance between STAT1 dimers activating transcription and STAT1 dimers repressing transcription.

Discussion

In this study, we identified BAL1/ARTD9 acting as a novel IFNγ-specific oncogenic survival factor in high-risk DLBCL. We further showed that constitutive overexpression of BAL1 in DLBCL tightly associates with intrinsic IFNγ signaling and constitutive nuclear activity of STAT1. BAL1 counteracts the IFNγ-dependent anti-proliferative and pro-apoptotic IFNγ–STAT1–IRF1–p53 axis while concomitantly activating IFNγ/STAT1-dependent (i.e. IRF2-mediated) and IFNγ/STAT1-independent (i.e. BCL6/BCL2-mediated) anti-apoptotic-pro-survival pathways. As a consequence, overexpression of BAL1 in HR-DLBCL mediates cell proliferation chemo-resistance and survival in high-risk DLBCL. The observed inhibition of tumor suppressor IRF1 and simultaneous upregulation of the proto-oncogenes *IRF2*, *BCL2* and *BCL6* by BAL1 strongly correlates with the phenotype of high risk DLBCL *in vitro* and the clinical outcome of HR-DLBCL (Abramson and Shipp, 2005; Monti et al., 2005).

IRF1 mediates anti-proliferative and pro-apoptotic effects in cancer cells in a context-dependent and cell-type-specific manner (Choo et al., 2006). In the absence of antagonistic regulatory factors, increased expression and activation of IRF1 inhibits the expression of pro-survival members of the BCL2 family and simultaneously induces the expression and activation of pro-apoptotic and anti-proliferative gene products, including p53, p21 and CASP3 (Choo et al., 2006; Schwartz et al., 2011; Shi et al., 2011). IRF2, an antagonist of IRF1 is known to act as an oncogene product in various types of cancer and when overexpressed in cancer, IRF2 can abolish the tumor suppression function of IRF1 (Choo et al., 2006). Although loss of IRF1 alone is not associated with spontaneous tumor development in mice, it greatly increases tumor susceptibility in combination with loss of other tumor suppressor proteins such as p53 (Nozawa et al., 1999). On the other hand, it has been previously demonstrated that BCL6 can also suppress both, the basal and the induced expression levels of tumor suppressors p53 and the cyclin-dependent kinase inhibitor p21 (Phan and Dalla-Favera, 2004; Saito et al., 2009). Loss or mutation of p53 are observed in about 20% of patients with high risk DLBCL and have statistically significant negative impact on progression-free survival (Stefancikova et al., 2011). Loss or mutation of p53 is associated with a shorter survival after R-CHOP treatment (Stefancikova et al., 2011). Thus the observed BAL1-mediated downregulation of tumor-suppressors IRF1 and simultaneous upregulation of BCL6 represents another molecular mechanism inactivating the p53/IRF1 pro-apoptotic pathway in high-risk DLBCL-expressing wild-type p53.

Remarkably, our study provides the first evidence that BAL1 not only blocks IFNγ–STAT1–IRF1-mediated apoptosis and inhibition of growth, but also reverses the chemo-resistance in high-risk DLBCLs. Our results also strongly indicate that STAT1 acts as an oncogene in high-risk HR-DLBCL with an active host inflammatory response. This activity is at least partially mediated by BAL1. BAL1 facilitates the oncogenic functions of STAT1 by counteracting the pro-apoptotic IFNγ–STAT1–IRF1 axis. Moreover, together with STAT1β, BAL1 may negatively regulate a tumor suppressor network, thereby inducing a switch in STAT1 from a tumor suppressor to an oncogene. This also explains why constitutive IFNγ–STAT1 signaling does not lead to apoptosis but rather to survival and chemo-resistance in HR-DLBCL (Fig. 6). Recent studies showed that aberrant nuclear localization and activity of STAT1 leads to radio- and chemo-resistance in solid cancers (Khodarev et al., 2004; Khodarev et al., 2009; Khodarev et al., 2012; Stronach et al., 2011; Weichselbaum et al., 2008).

Strikingly, our studies also demonstrate that BAL1 interacts with the IFNGR complex and enhances tyrosine phosphorylation

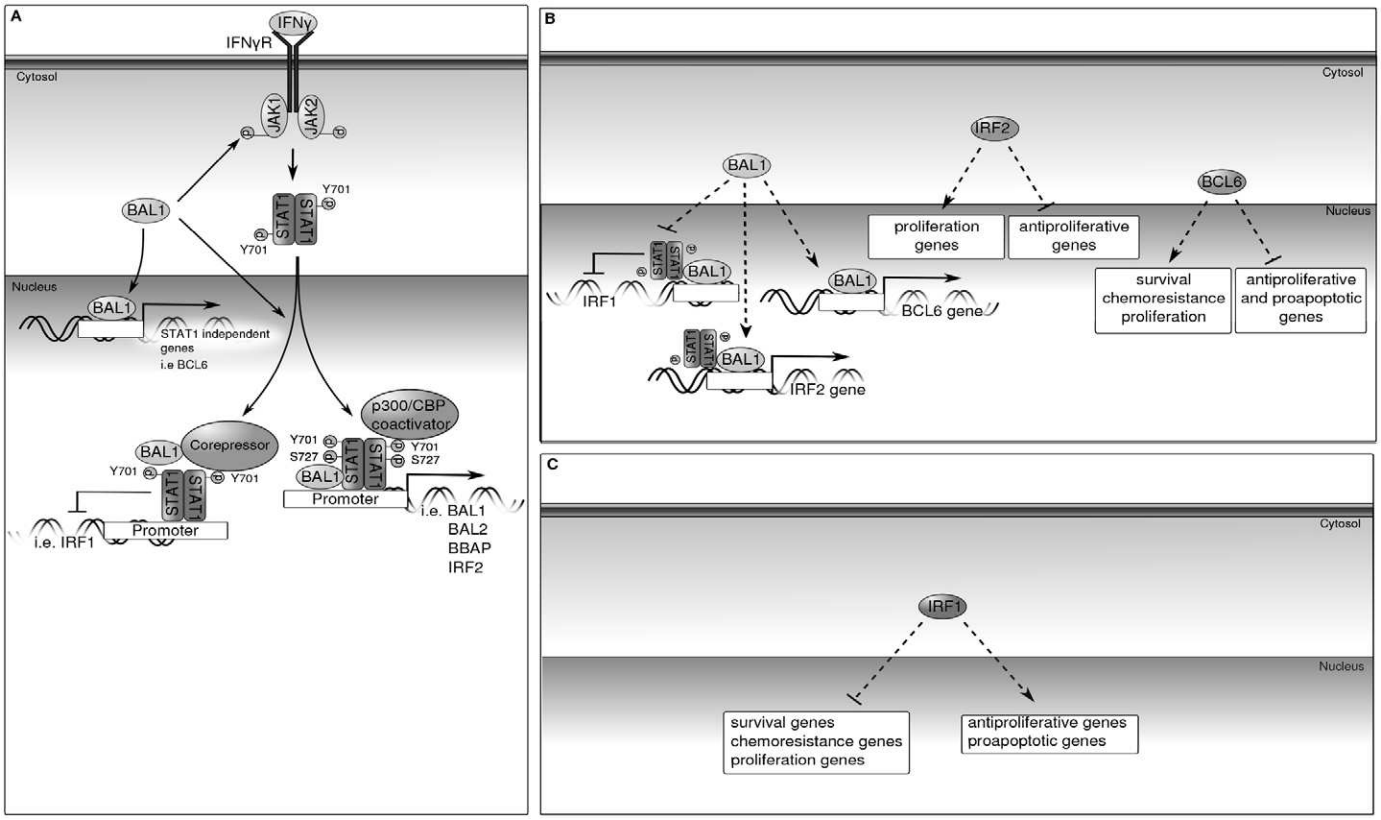

Fig. 6. Model for the BAL1-mediated oncogenic switch in STAT1 in HR-DLBCL. (**A**) Constitutively active IFNγR-JAK1/2-STAT1 signaling in HR-DLBCL causes overexpression of BAL1, which in turn further stimulates the phosphorylation of STAT1 on Y701 and subsequently the enhanced expression of STAT1α-dependent proto-oncogenes (i.e. *BBAP*/*BAL1*, *BAL2*, *IRF2*). Conversely, BAL1 together with STAT1β represses the transcriptional activation of tumor suppressor IRF1. In addition, overexpression of BAL1 also enhances the expression of STAT1-independent proto-oncogenes (i.e. *BCL6*). (**B**) Overexpression of BAL1 prevents intrinsic and extrinsic IFNγ-STAT1-IRF1/p53-mediated cell death pathways while simultaneously enhancing the STAT1-dependent IRF2-mediated proliferation and STAT1-independent BCL6–BCL2-mediated survival pathways. As a consequence BAL1 induces an switch in STAT1 from a tumor suppressor to an oncogene and mediates proliferation, survival and chemo-resistance in HR-DLBCL. (**C**) Inactivation of BAL1 in HR-DLBCL associated with constitutively active STAT1 signaling reactivates the anti-proliferative and pro-apoptotic IFNγ-STAT1-IRF1/p53 axis and reverses chemo-resistance in HR-DLBCL.

of both STAT1 isoforms, STAT1α and STAT1β, on Y701, and their subsequent nuclear translocation, thereby promoting the nuclear accumulation of the antagonistically acting and transcriptionally repressive isoform STAT1β. Thus, BAL1 influences the nuclear activities of the transcriptionally repressive isoform STAT1β, tipping the antagonistic balance between STAT1 dimers activating transcription and STAT1 dimers repressing transcription. However, the exact molecular mechanism(s) underlying the isoform- and promoter-specific regulation of STAT1 by BAL1 remain to be elucidated in future studies. Unfortunately, the specific antagonistic nuclear activities of STAT1α and STAT1β, and their distinct phosphorylated and unphosphorylated forms in particular, are in general not very well investigated yet.

In contrast, *BCL6* is not likely to be a direct target gene of STAT1, so the observed BAL1-mediated stimulation of BCL6 expression might be caused by another unknown mechanism. BCL6 overexpression is mediated through multiple mechanisms in DLBCL: translocation, hypermutation of its promoter, or inactivation of FBXO11, which results in increased levels and stability of BCL6 (Duan et al., 2012). Transcriptional upregulation of *BCL6* by BAL1, therefore, represents an additional molecular mechanism for overexpression of BCL6 in DLBCL. Moreover, the observed concomitant overexpression of BCL2 and BCL6 in SUDHL-7 cells also indicates that BAL1 could block BCL6-mediated repression of the *BCL2* gene, which is frequently disrupted in DLBCL (Saito et al., 2007; Saito et al., 2009). However, the exact STAT1-independent molecular mechanisms involved in BAL1 stimulation of the transcriptional activation of *BCL6* and *BCL2* have to be thoroughly investigated in future.

Finally our study provides the first evidence for an IFNγ-dependent STAT1–BAL1–BCL6-mediated anti-apoptotic-, pro-survival-regulatory circuit in HR-DLBCL and explains why STAT1 could function as an oncogene in a subset of HR-DLBCL. In addition, our observations could also provide a molecular mechanism for the risk-related activity of BAL1 in HR-DLBCL subsets without constitutive active STAT1 signaling. BAL1 could be directly involved in editing or inhibiting the IFNγ-dependent host immune response against HR-DLBCL through the termination of IFNγ-mediated gene expression and inhibition of the extrinsic IFNγ-induced

anti-proliferative and pro-apoptotic STAT1–IRF1–p53 axis. The observed macrodomain and ADP-ribosylation-mediated interaction between BAL1 and STAT1 as well as the BAL1-mediated upregulation of BAL2/ARTD8 also indicates a regulatory cross talk between BAL1 and other active members of the ARTD family such as BAL2/ARTD8 or ARTD10 in these processes. BAL2/ARTD8 is a macrodomain-containing ARTD family member and an active mono-ADP-ribosyltransferase mediating survival in c-Myc-driven Burkitt lymphoma-like tumor cells *in vivo* (Cho et al., 2009a). BAL2/ARTD8 has been suggested to function as a STAT6-specific co-regulator of IL-4-mediated gene expression and has been suggested to be involved in mediating IL-4-induced proliferation and protection of B-cells against apoptosis following irradiation or growth factor withdrawal (Cho et al., 2009b; Goenka and Boothby, 2006; Goenka et al., 2007).

Together, our studies further strengthen the hypothesis that BAL1 may serve as a novel potential drug target for treatment of high-risk chemo-resistant diffuse large B-cell lymphoma. The combination of classic therapeutic drugs with novel drugs targeting STAT1 or the macrodomains of BAL1/ARTD9 might be a strategy to increase the sensitivity of HR-DLBCL towards classic therapy, and thus pave the way to develop novel therapeutic strategies for the remainder of DLBCL patients suffering from aggressive chemo-resistant high-risk host response variants of DLBCL.

Material and Methods

Cell culture, transfections, luciferase reporter assays and generation of stable cell lines

The human lung carcinoma cell line A549, 293HEK and DLBCL cell lines were cultured as described previously (Hassa et al., 2005; Saito et al., 2009). The DLBCL cell lines SUDHL2, SUDHL4, SUDHL6, SUDHL7, U2932, OCI-Ly1, OCI-Ly3 and OCI-Ly10 were provided by Dr Riccardo Dalla-Favera (Columbia University, NY, USA), Dr Jose Martinez-Climent (Spanish National Cancer Research Centre, Madrid, Spain) and Dr Louis Staudt (National Institutes of Health, Bethesda, MD, USA). 293HEK were purchased from ATCC. Transfections of cells with plasmid DNA (for reporter assays and generation of stable cell lines) were performed with Fugene HD, Extreme gene 9 and HP transfection reagents (Roche Applied Science) according to the manufacturer's protocols. Stable cell lines were generated using a Piggyback transposon and a BIC-miR155-precurser-RNA based shRNA-expressing system. Piggyback-transposed cells were selected with puromycin (500 ng/ml). Transfections of siRNA oligos (40 pmol siRNA/transfection) were performed with Lipofectamine RNAimax reagent (Invitrogen) or Extreme gene siRNA (Roche Applied Science) according to manufacturers' protocols. Luciferase reporter assays were performed as previously described (Hassa et al., 2005) and according to manufacturer's protocols (Promega) using the Dual Luciferase assay kit (Promega) and a TECAN infinite M200 luminometer (Tecan Systems).

Plasmids

Human BAL1/ARTD9 cDNA was amplified by PCR from a B-cell lymphoma cDNA library and cloned into the corresponding expression vectors (pcDNA-HA-, pPiggyBac-EF1aprom-HA-BAL1) using *Nhe*I–*Not*I or *Not*I, respectively. BAL1-domain deletion and GST-fusion constructs were generated by PCR and cloned into the *Nhe*I–*Not*I or *Eco*RI–*Not*I sites of pcDNA-HA- and pETM-GST-MCS1, respectively. All constructs and full-length cDNA sequences were verified by sequencing. All empty basic Piggyback transposon vectors and expression vectors for the latest version of Piggyback transposases were either purchased from System Biosciences (SBI) Inc. (Mountain View, CA, USA) or provided by Dr Allan Bradley (Wellcome Trust Sanger Institute, UK). The BIC-mir155 vector system (Chung et al., 2006) was provided by Dr David L. Turner (Molecular and Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, USA). The siRNA and shRNA/DNA oligos were purchased from Qiagen. The corresponding siRNA and shRNA sequences are listed in supplementary material Table S1. FLAG–STATs were purchased from Addgene. *hIRF1*-prom-luciferase reporter vectors were a gift from Dr R. Pine (Public Health Research Institute, Newark, NJ, USA).

Reagents

Human recombinant IFNγ was purchased from PeproTech, doxorubicin and etoposide were purchased from Sigma. Tosyl-//activated Dynabeads were purchased from Invitrogen. ADP ribose was purchased from Sigma. High-performance glutathione–Sepharose and Ni-Sepharose were purchased from Amersham Biosciences.

Gene expression analysis

Real-time qPCR analysis was performed essentially as described previously (Guetg et al., 2012). Total RNA was isolated using Trizol (Invitrogen) or Tri-Reagent (MRC Inc.) according to manufacturers' protocols. RNA was subsequently reverse-transcribed using the 'High-Capacity cDNA Reverse Transcription kit' (Applied Biosystems) according to manufacturer's protocols. Real-time qPCR was performed using the Rotor-Gene 3000 (Corbett Life Science, now Qiagen) and SYBR Green kit (Bioline) according manufacturers' protocols using the primers listed in supplementary material Table S2. Mean values ± s.e.m. were calculated and plotted as graphs with GraphPad Prism (GraphPad Software).

Chromatin immunoprecipitation

Cells were collected and crosslinked with 1% formaldehyde as described previously (Covic et al., 2005). Chromatin fragmentation was achieved with the Bioruptor (Diagenode). Antibodies were incubated with crosslinked chromatin overnight at 4°C and collected with Protein-A agarose/salmon sperm DNA (Millipore) for 3 hours. After reversal of the crosslinking and digestion with proteinase K, DNA was extracted and measured by real-time PCR using SYBR Green and the Rotor-Gene 3000 (Corbett Life Science/Qiagen).

Expression, purification of recombinant proteins

Recombinant HIS–GST–BAL1 fusion proteins were expressed in *E. coli* strains BL21 DE3-Rosetta-II, BL21 DE3-Rosetta-II-Tuner and BL21-ArcticExpress-Rosetta-II as described previously (Timinszky et al., 2009). All purified proteins were analyzed using Coomassie Blue staining and confirmed by western blot analysis using the corresponding antibodies.

Interaction assays, immunoblot analysis and immunofluorescence microscopy

Membrane, cytoplasmic, nuclear and whole-cell extracts were prepared as described previously (Cunningham et al., 2003; Dignam et al., 1983; Hassa et al., 2005; Okada et al., 2008; Sen et al., 2011) with minor modifications: membrane extraction buffer contain 50 mM HEPES (pH 7.9) 150 mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 1 mM EDTA, 1 mM EGTA and cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche). For immunoprecipitation, membrane and cytoplasmic extract fractions were re-mixed. Co-immunoprecipitation and GST pull-down assays were performed as described previously (Hassa et al., 2005; Owen et al., 2005; Timinszky et al., 2009), except that the antibodies used for immunoprecipitation were covalently coupled to tosyl-activated Dynabeads (Invitrogen) according to the manufacturer's protocols. Immunoprecipitation buffers contained 80 mM Tris (pH 7.05), 125 mM NaCl, 25 mM potassium acetate, 1.5 mM $MgCl_2$, 5% glycerol, 0.5% NP-40, 0.5 mM DTT, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 0.1 mM Na_3VO_4, and cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche). GST pull-down buffers contained 80 mM Tris (pH 7.05), 1.5 mM $MgCl_2$, 150 mM NaCl, 50 mM potassium acetate, 0.5% NP-40, 0.5 mM DTT, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 5% glycerol and cOmplete, EDTA-free Protease Inhibitor Cocktail (Roche). Immunoblot procedures and immunofluorescence microscopy were performed as described previously (Guetg et al., 2012; Hassa et al., 2005) using the following primary antibodies: monoclonal mouse anti-HA and mouse anti-FLAG antibodies (both Sigma), polyclonal rabbit anti-BAL1 antibodies (C-terminal; Millipore), monoclonal rabbit anti-STAT1, anti-pSTAT1(Y701), anti-pSTAT1(S727), anti-STAT2, anti-pSTAT2(Y690), anti-IRF2, anti-BLIMP1, anti-Casp3, anti-BCL2, anti-BAD, anti-BAD-S112, anti-PIM1, anti-PIM2, anti-PTBN1, anti-JAK1, anti-pJAK1, anti-JAK2, anti-pJAK2, anti-IFNGR1, anti-PTBN2 and anti-BCL-XL antibodies (RabMab, Epitomics), monoclonal rabbit anti-IRF1 (RabMab, Cell Signaling Technology), monoclonal mouse anti-tubulin, polyclonal rabbit anti-p53 and anti-BCL6 antibodies (Santa Cruz Biotechnology). The polyclonal rabbit anti-BAL2/ARTD8 antibody was a generous gift from Avraham Raz (Karmanos Cancer Institute, School of Medicine, Wayne State University, Detroit, Michigan 48201, USA) (Yanagawa et al., 2007). Immunofluorescently stained cells were analyzed by fluorescence microscopy on a Leica DMI6000B automated inverted research microscope system (Leica Microsystem). Composite images were generated using Adobe Photoshop software.

Flow cytometry analysis, survival and proliferation assays

FACS analysis was performed with the following PE-conjugated antibodies (Biolegend) according to manufacturer's protocols: PE anti-human CD119 (IFNγR α-chain) PE anti-human CD40, PE Syrian hamster IgG isotype control and PE mouse IgG2 isotype control antibodies. PE-positive and PE-negative cells were

sorted using a BD FACSVantage cell sorter flow cytometer (BD Biosciences). Cell viability and proliferation were assessed by Trypan Blue exclusion and by the WST1 assays (Roche Diagnostics) according to manufacturers' protocols. For the cell viability and proliferation assays cells were seeded at 0.2×10^6 cells/ml in six-well dishes 8 hours prior to initiation of treatment and then incubated in the presence of PBS, DMSO (mock-treated), etoposide (20 μM) or doxorubicin (2 μM) for 24 hours. After 24 hours the cultures were washed with medium prior to reseeding in fresh medium for continued culture (24, 48, 72 and 96 hours). Relative cell viability/proliferation and cell numbers are presented as means from three independent experiments performed in triplicate ± standard deviations.

Analysis of tissue samples

In total, 250 formalin-fixed and paraffin-embedded tissue samples were analyzed, which consisted of 12 tonsils with reactive lymphoid hyperplasia and 238 diffuse large B-cell lymphomas (DLBCL). Histopathological diagnosis was rendered according to the World Health Organization classification. Two cores of 0.1 cm diameter were punched out of each sample of the respective donor blocks and transferred to Tissue-Micro-Array recipient blocks, from which 3 μm thick sections were cut and mounted on Superfrost slides (Menzel Glaser). The primary antibodies used were anti-pSTAT1(Y701) (rabbit polyclonal, Cell Signaling Technology, dilution 1:25 in Ventana Buffers), anti-pSTAT1(S727) (rabbit monoclonal, Epitomics Inc., dilution 1:400 in Ventana Buffers) and anti-IRF2 (rabbit monoclonal, Epitomics Inc., dilution 1:50 in Ventana Buffers). Staining was performed with the Ventana Discovery Ultra automated staining system (Ventana Medical Systems Inc.), operated according to the manufacturer's instructions and using solely Ventana reagents during the procedure. Detection was performed with the ChromoMap DAB detection kit using Ultra Map Anti Rb HRP. Negative controls were performed by omission of the primary antibodies. Slides were counterstained with Hematoxylin, dehydrated and mounted. Staining intensity was assessed with a light microscope, allocating a semi-quantitative score to each core. For all three markers, the approximate percentage of stained nuclei was recorded. The scoring was undertaken as follows: $<10\%$ was scored as 1, 10–50% as 2, 50–80% as 3 and $>80\%$ as 4; negative staining was scored as 0. Scoring was omitted for cores not containing appropriate tissue or in cores with artifacts resulting from crushing (see also supplementary material Tables S3, S4).

Acknowledgements

We are grateful to Drs R. Dalla-Favera (Columbia University, New York, NY, USA), L. Staudt (National Institutes of Health, Bethesda, MD, USA), I. Lossos (University of Miami, FL, USA.), J. Martinez-Climent (Spanish National Cancer Research Centre, Madrid, Spain), U. Novak (Inselspital, Bern, Switzerland), M. Thome (University of Lausanne, Epalinges, Switzerland), R. Pine (Public Health Research Institute, Newark, NJ, USA), J. Pavlovic (University of Zurich, Switzerland), A. Bradley (Wellcome Trust Sanger Institute, UK) D.L. Turner (University of Michigan, Ann Arbor, USA) and A. Raz (Wayne State University, Michigan USA) for providing cells, plasmids and reagents. We also thank all the members of the Institute of Veterinary Biochemistry and Molecular Biology (University of Zurich, Switzerland) for helpful advice and discussions.

Author contributions

R.C., H.C.W., S.B.B. and P.O.H. designed the experiments and analyzed results. R.C., H.C.W., S.B.B. and P.O.H. performed the research. M.T., E.H. and M.B. performed the evaluation of clinical samples. R.C. and P.O.H. wrote the paper. P.O.H. designed and supervised the research study. H.C.W. and S.B.B. contributed equally to the paper. All the authors read and corrected the manuscript.

Funding

This work was supported by the Swiss National Science Foundation [grant number SNF-31003A_125190 to P.O.H., R.C. and H.C.W.]; Novartis Foundation for Medical-Biological Research [grant number 10C63 to P.O.H.]; and by the Kanton of Zurich (to P.O.H., M.T., E.H. and M.B.).

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.118174/-/DC1

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